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# Glycoprotein nature of $D_2$ dopamine receptors

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The glycoprotein nature of the ligand binding subunit of photoaffinity-labeled striatal $D_2$ receptors was investigated. Upon photolysis, [$^{125}$I]*N*-azidophenethylspiperone covalently incorporated into a major band of $M_r$ 94000 with an appropriate pharmacological profile for $D_2$ receptors as assessed by autoradiography following SDS-polyacrylamide gel electrophoresis. The exoglycosidase, neuraminidase, altered the electrophoretic mobility of the 94 kDa labeled band to 54 kDa with a slight modification in the binding affinity of [$^3$H]spiperone. Endoglycosidase treatment (glycopeptidase-F) produced a further increase in the mobility of the 94 kDa peptide to ~43 kDa. A smaller specifically photolabeled $D_2$ receptor peptide of 34 kDa does not contain terminal sialic acid but is an *N*-linked glycoprotein as assessed by lectin affinity chromatography and susceptibility to digestion by glycopeptidase-F to a peptide of ~23 kDa.

Dopamine $D_2$ receptor; Photoaffinity labeling; Neuraminidase; Glycopeptide-*N*-glycosidase; SDS-PAGE

## 1. INTRODUCTION

The dopamine $D_2$ receptor has been identified and classified on the basis of its ability to inhibit adenylate cyclase activity and bind or respond to specific dopaminergic agonists and antagonists (reviews [1–3]). The recent development of specific photoaffinity labels for $D_2$ dopamine receptors, by us and others (see [3]) now allows for the analysis of these membrane proteins at the molecular level. Numerous proteins including many neurotransmitter receptors are glycoproteins [4–9]. In this communication, we demonstrate the glycoprotein nature of the striatal $D_2$ receptor ligand binding subunit by enzymatic digestion of photolabeled receptor proteins.

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*Abbreviations:* [$^{125}$I]NAPS, [$^{125}$I]*N*-azidophenethylspiperone; WGA, wheat germ agglutinin; PAGE, polyacrylamide gel electrophoresis; PNGase F, glycopeptidase-F

## 2. MATERIALS AND METHODS

[$^{125}$I]NAPS (2200 Ci/mmol) was purchased from New England Nuclear and [$^3$H]spiperone (85 Ci/mmol) from Amersham. Neuraminidase (purified type X from (*Clostridium perfringens*), $\alpha$-mannosidase (from jack bean), and Nonidet P-40 were from Sigma. Purified glycopeptidase-F (peptide-N$^4$ [*N*-acetyl-$\beta$-glucosaminyl]asparagine amidase) from *Flavobacterium meningosepticum* was from Boehringer-Mannheim. All other materials were from sources described in [10,11].

### 2.1. *Membrane preparation*

Striata, dissected from partially thawed canine brains (Pel Freez, Rogers, AR) were Teflon-glass homogenized in 20 vols of 25 mM Tris-HCl buffer containing 250 mM sucrose and the following protease inhibitors: 20 mM EDTA, 5 $\mu$g/ml leupeptin, 15 $\mu$g/ml benzamidine, 5 $\mu$g/ml soybean trypsin inhibitor and 1 mM PMSF (pH 7.4 at 4°C). Homogenates were centrifuged at 600 × *g* for 10 min and the supernatant recentrifuged at 48000 × *g* for 20 min. The resulting pellet was resuspended in 25 mM Tris-HCl buffer containing 120 mM NaCl and protease inhibitors to yield a final protein concentration of approx. 2 mg/ml.

### 2.2. *Photoaffinity labeling*

Membranes (1 ml) were routinely incubated (in the dark) with [$^{125}$I]NAPS (50 pM final concentration) in the absence or presence of 1 $\mu$M (+)-butaclamol or other dopaminergic agents (as indicated) for 90 min at 22°C in a final volume of 10 ml at ~50 pM $D_2$ receptor and processed for photolysis as described



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[12]. Samples (1 ml) were irradiated for 35 s as described [10], transferred to 1.5 ml Eppendorf microfuge tubes and sedimented at 12000 × *g* for 10 min.

2.3. *Exoglycosidase treatments*

Photoaffinity-labeled membranes were washed twice in 30 ml of 100 mM sodium acetate buffer (pH 5 at 22°C) containing 100 μM PMSF. Pellets were resuspended in buffer (0.5–1.0 mg/ml) and incubated with neuraminidase (up to 2 U/ml) at 37°C for the indicated time periods. For α-mannosidase treatment, photolabeled receptors were washed in 50 mM sodium citrate buffer (pH 4.5 at 22°C) containing 100 μM PMSF and incubated with up to 12 U/ml of α-mannosidase for 24 h at 25°C. Following incubation, membranes were pelleted, washed once with 25 mM Tris-HCl buffer and prepared for SDS-PAGE as described in [10].

2.4. *Endoglycosidase treatment*

Photolabeled and neuraminidase-treated membranes were washed twice in 200 mM sodium phosphate buffer (pH 8.1 at 25°C) containing 2.5 mM EDTA, and incubated with 0.5% SDS and 100 mM β-mercaptoethanol for 60 min at 22°C. Aliquots (30 μl, ~500 μg protein) were incubated with Nonidet P-40 (1.25% final concentration), PNGase-F (up to 60 U/ml) and 50 mM EDTA for 20 h at 37°C in a volume of 60 μl (final concentration of SDS < 0.17%). Samples were centrifuged at 12000 × *g* for 10 min, the supernatant aspirated and samples added to SDS-sample buffer (containing 20% SDS) for 120 min at 22°C.

2.5. *$D_2$ receptor solubilization*

Photolabeled membranes were solubilized in 1% digitonin (2 ml) and applied to a 2 ml WGA-Sepharose column at 4°C as described in [10,11]. Absorbed receptors were specifically eluted with 150 mM *N*-acetylglucosamine. Samples were then lyophilized and processed for electrophoresis as described above.

2.6. *Ligand binding*

[$^3$H]Spiperone binding to $D_2$ receptors and computer assisted analysis of all binding data (LIGAND) were as described [10,11].

2.7. *SDS-PAGE and autoradiography*

Prior to electrophoresis, samples were dissolved in SDS-sample buffer as described [10] for 60–120 min at 22°C. Aliquots (~100–300 μg protein) were loaded on 1.5 mm-thick slab gels containing a 10 or 12% acrylamide separating gel and electrophoresed overnight. Gels were dried, exposed to Kodak XAR-5 film with intensifying screen for 20 h at −70°C and manually developed with Kodak D-19 developer. Prestained molecular mass standards (Sigma) were used to visualize protein migration.

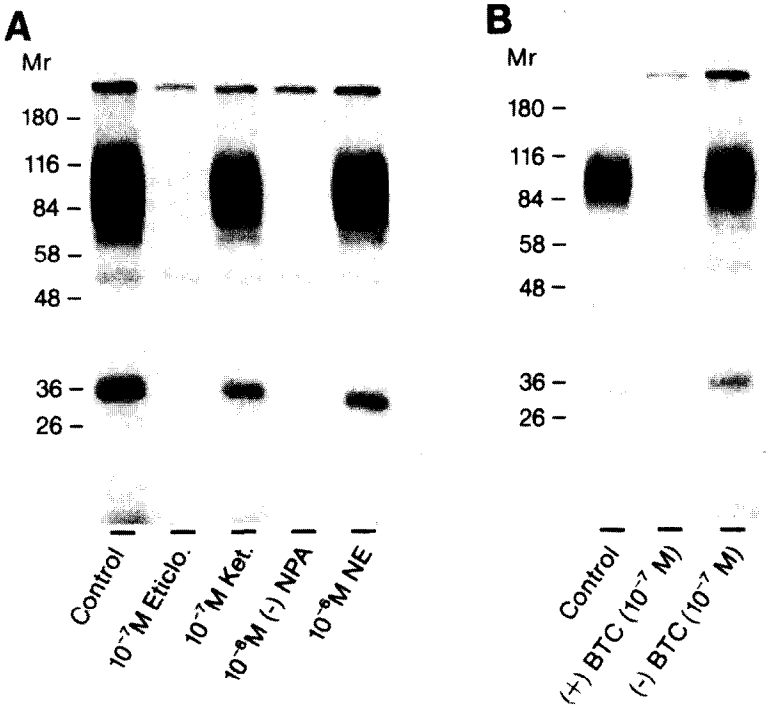


Fig.1. Pharmacological specificity of [$^{125}$I]NAPS photoincorporation into canine striatal membranes. Striatal homogenates were incubated with [$^{125}$I]NAPS alone or with dopaminergic agents, photolyzed and prepared for electrophoresis on a 10% acrylamide gel as described in section 2. The results shown are identical with at least three other experiments. Molecular mass standards are shown on the left (× 1000). Eticlo., eticlopride; Ket., ketanserin; NPA, *N*-propylnorapomorphine; NE, noradrenaline; BTC, butaclamol.

## 3. RESULTS AND DISCUSSION

As illustrated in fig.1 (A and B), [$^{125}$I]NAPS photoincorporated into a striatal peptide of $M_r$ 94000 as assessed by autoradiography following SDS-PAGE. The broad band at $M_r$ 94000 represents the ligand binding subunit of the $D_2$ receptor since photoaffinity labeling is stereoselectively blocked by dopaminergic antagonists and agonists with an appropriate pharmacological profile for $D_2$ receptors. In addition, a peptide of $M_r$ ~36000–34000 revealed a similar pharmacological profile for $D_2$ receptors. The stoichiometry of labeling of the 94 kDa and 34 kDa peptide was ~3:1. Whether the peptide at $M_r$ 34000 represents a proteolytic degradation product of the peptide at $M_r$ 94000 is still unknown, although under our assay conditions this peptide is always seen either in the absence or presence of protease inhibitors (not shown). Minor specifically labeled peptides (e.g. $M_r$ 66000) were inconsistently observed as well as a non-specifically labeled protein at $M_r$ 51000.

The carbohydrate nature of the ligand binding subunit(s) of the $D_2$ receptor was assessed by the exoglycosidase, neuraminidase, which specifically cleaves terminal sialic acids. As seen in fig.2, neuraminidase altered the electrophoretic mobility of the peptide at $M_r$ 94000 to $M_r$ ~54000 without affecting the migration of the photolabeled peptide at $M_r$ 34000. At present, it is unclear whether the change in the migration pattern of the 94 kDa peptide induced by neuraminidase is the result of the cleavage of a large quantity of receptor-associated sialic acids or simply due to the removal of negatively charged carbohydrate moieties interfering with the protein denaturing effects of SDS. In any event, it should be noted that not all of the photolabeled protein at $M_r$ 94000 is digested by neuraminidase. Both the 94 kDa and 34 kDa labeled peptides are glycoproteins since they absorb to and specifically elute from WGA-Sepharose (fig.3) prior to or following neuraminidase treatment suggestive of a poly(*N*-acetyllactosamine)-type glycopeptide [13]. Treatment of photolabeled membranes with up to 12 U/ml of $\alpha$-mannosidase did not alter the mobility of SDS-PAGE-separated peptides ($M_r$ 94000 and 34000), although a minor peptide of $M_r$ ~58000 was observed (not shown). These data suggest that the ligand binding subunit of $D_2$ receptors contains terminal sialic acid with few terminal high mannose chains and is consistent with the observation that solubilized $D_2$ receptors absorb to and are specifically eluted from WGA-Sepharose but not concanavalin-A columns (see above and [10,14,15]).

Following neuraminidase, the peptide at $M_r$ 54000 appears to bind the selective $D_2$ receptor antagonist, [$^3$H]spiperone (fig.4B) in a saturable manner without significantly affecting receptor density (control: 5.5 ± 0.2 pM; treated 5.2 ±

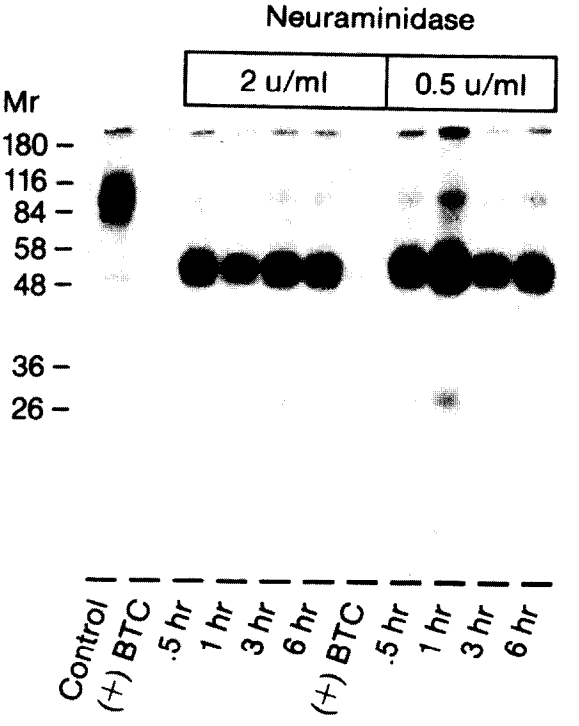


Fig.2. Effect of neuraminidase on the SDS-PAGE migration pattern of photolabeled $D_2$ receptors. [$^{125}$I]NAPS-labeled membranes were prepared for enzyme treatment as described in section 2. Neuraminidase was added at the indicated concentrations for various periods of time at 37°C. Control samples were incubated at 37°C for 6 h in the absence or presence of 1 $\mu$M (+)-butaclamol. Included in this gel is a lane demonstrating non-specific labeling for neuraminidase-treated receptors. Following treatment, membranes were prepared and subjected to SDS-PAGE as described in section 2 using a 12% acrylamide gel. Abbreviations used are as in fig.1.

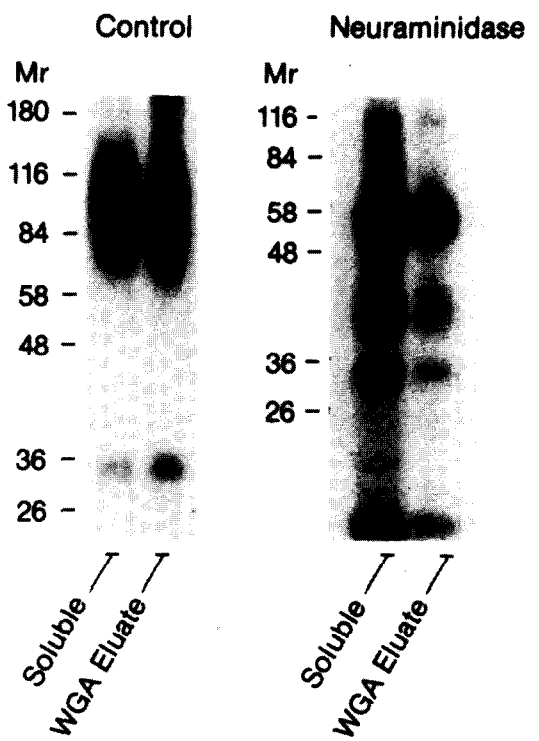


Fig.3. Effect of neuraminidase on WGA-lectin affinity eluates. [$^{125}$I]NAPS-labeled receptors were prepared for enzyme treatment as described in section 2. Photolabeled membranes were incubated without (control) or with 2 U/ml neuraminidase for 30 min at 37°C. Membranes were washed, solubilized with 1% digitonin and centrifuged. The supernatant (2 ml) was applied to a column of WGA-Sepharose equilibrated with 50 mM Tris-HCl and 0.1% digitonin at pH 7.4 at 4°C, recycled and washed with 20 ml of the same buffer. Absorbed protein was eluted by the addition of 150 mM *N*-acetylglucosamine (WGA eluate) in 0.05% digitonin. Equal amounts of protein (~250 $\mu$g) from solubilized and eluted samples were lyophilized and subjected to electrophoresis on 10% (control) or 12% (neuraminidase) gels and autoradiography.

0.4 pM) as determined by saturation analysis. The affinity of [$^3$H]spiperone for the peptide at $M_r$ 54000 was reduced by ~2–3-fold from 110 ± 6 pM to 288 ± 12 pM. It is of interest to note that the brief exposure of the membrane (20 min) to pH 5.0 irreversibly inactivated ~70% of the original receptor population ($B_{max}$ ~20 pM). This effect was not prevented by prior occupancy of the receptor by $D_2$ receptor antagonists such as (+)-butaclamol or spiperone.

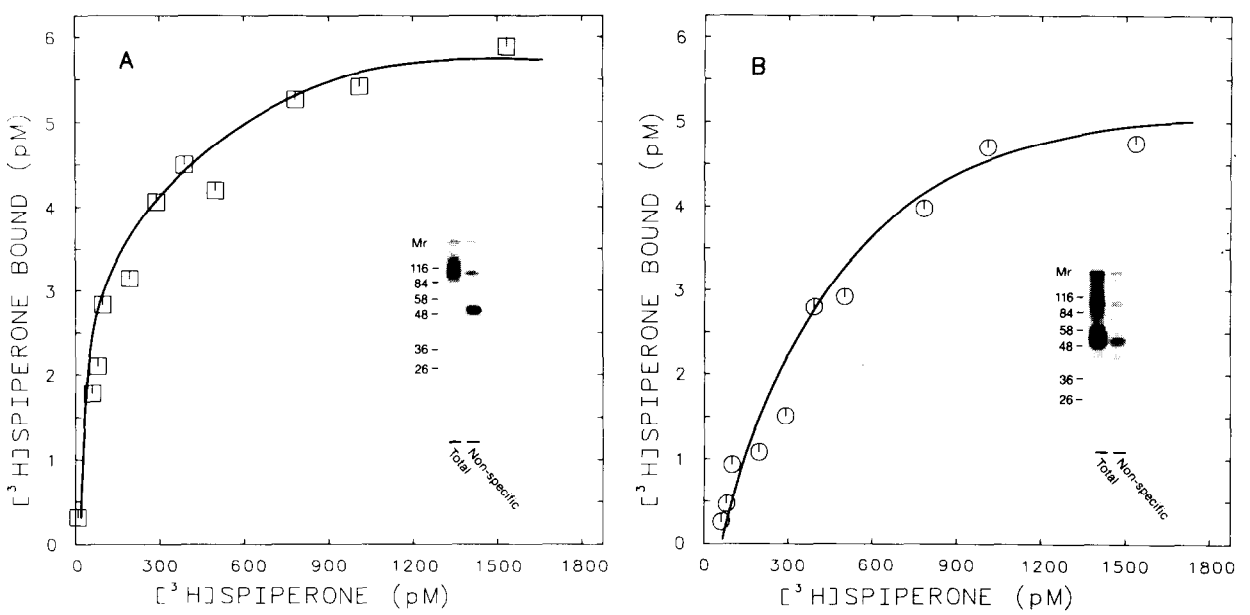


Fig.4. Effect of neuraminidase on [$^3$H]spiperone binding activity. Striatal membranes were prepared for enzyme treatment as described in section 2. Neuraminidase (B) was added at a concentration of 2 U/ml and incubated for 20 min at 37°C. Control membranes (A) were treated in an identical fashion but without neuraminidase. Following treatment, membranes were extensively washed in Tris-HCl buffer containing 120 mM NaCl, pH 7.4, at 22°C and assayed for $D_2$ receptor activity with [$^3$H]spiperone (10–1800 pM) as described. Data were analyzed by LIGAND and are representative of two similar experiments. (Inset) Control and neuraminidase-treated tissue were incubated with [$^{125}$I]NAPS (50 pM), in the absence or presence of (+)-butaclamol (1 $\mu$M), photolyzed and prepared for SDS-PAGE and autoradiography as described in section 2.

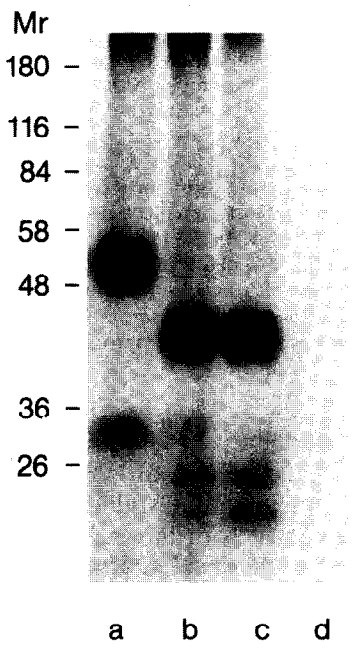


Fig.5. Sequential neuraminidase-PNGase-F treatment. [$^{125}$I]NAPS-labeled membranes were prepared for enzyme treatment as described. Aliquots were incubated with PNGase-F for 20 h at 37°C as follows. Lanes: a, control, 2 U/ml neuraminidase; b, 2 U/ml neuraminidase and 10 U/ml PNGase-F; c, 2 U/ml neuraminidase and 60 U/ml PNGase-F; d, same as lane c except photolabeling in the presence of 1 $\mu$M (+)-butaclamol. Following treatment, aliquots were prepared for SDS-PAGE and autoradiography as described.

Complete removal of *N*-linked carbohydrate by sequential neuraminidase and endoglycosidase treatment with PNGase F revealed that the major photolabeled peptide of $M_r$ 94000 representing the ligand binding subunit of the $D_2$ receptor resides in a peptide of $M_r$ ~43000 (fig.5). The 34 kDa labeled fragment following PNGase F treatment had migrated to ~23 kDa with an intermediate labeled protein of 26 kDa. Similar results were obtained with endoglycosidase-F (NEN) at pH 8.0 (not shown). Whether the polypeptide at $M_r$ 26000 represents an incompletely deglycosylated receptor fragment or a proteolytic breakdown product is unknown at present.

In summary, these data indicate that the ligand binding subunit of $D_2$ receptors is associated with sialic acid/acetylglucosamine carbohydrate chains and suggests that the molecular mass of the $D_2$-binding subunit is greatly overestimated due to receptor-associated carbohydrate. Moreover, the differential susceptibility of the peptides at $M_r$ 94000 and 34000 to digestion by exo- and endoglycosidases suggests that the $D_2$ receptor is composed of two distinct glycosylated protein subunits and is possibly a dimer of $M_r$ 23000 ($\times$ 2). The functional significance of these receptor-associated carbohydrate groups for the expression and maintenance of $D_2$ receptor activity is unknown at present.

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